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## **Transcriptional landscape of human keratinocyte models exposed to 60-GHz millimeter-waves**

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## Abstract

The use of millimeter waves (MMW) will exponentially grow in the coming years due to their future utilization in 5G/6G networks. The question of possible biological effects at these frequencies has been raised. In this present study, we aimed to investigate gene expression changes under exposure to MMW using the Bulk RNA Barcoding and sequencing (BRB-seq) technology. To address this issue, three exposure scenarios were performed aiming at: i) comparing the cellular response of two primary culture of keratinocytes (HEK and NHEK) and one keratinocyte derivate cell line (HaCaT) exposed to MMW; ii) exploring the incident power density dose-effect on gene expression in HaCaT cell line; and, iii) studying the exposure duration at the new ICNIRP exposure limit for the general population. With the exception of heat effect induced by high power MMW (over 10 mW/cm<sup>2</sup>), those exposure scenarios have not enabled us to demonstrate important gene expression changes in the different cell populations studied. Very few differentially genes were observed between MMW exposed samples and heat shock control, and most of them were significantly associated with heat shock response that may reflect small differences in the heat generation. Together these results show that acute exposure to MMW has no effects on the transcriptional landscape of human keratinocyte models under athermal conditions.

Keywords: millimeter waves; BRB-seq; keratinocytes; gene expression

### Highlights

Millimeter waves have no effect on Keratinocyte models transcription

The only recognized effect is the heat effect associated to MMW

The only effects are due to the heat generation process

## 1. Introduction

The requirement of new kind of frequency for technological development are increasing. Among all radiofrequencies, millimeter waves that are used in the 5G/6G wireless networks, are a range of frequencies between 30 GHz and 300 GHz. Their peculiar physical properties make them of interest for telecommunication considering their short latency (<1 ms), high speed data transfer (multi-Gbps), and reduced size of devices (Bautista et al., 2019). Even if in the 5G deployment these frequencies are not yet widely deployed, the future technological development will surely use these peculiar frequencies. Question on their potential adverse associated risks are nevertheless still pending (Simkó and Mattsson, 2019). Up to now, the main link with pathology comes from epidemiologic studies that have relied the occurrence of acoustic neurinome and some brain cancer with large use of mobile phone, however these results concern only lower part of radiofrequencies. After intense debate on results (Hardell et al., 2013; INTERPHONE Study Group, 2011, 2010), IARC classified electromagnetic waves as possibly carcinogenic (Risk 2B) (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2013). However, no convergent information and no mechanisms are really available on MMW and cancer promotion particularly.

Among all possible mechanisms potentially disturbed by the MMW, we have intensively explored the gene expression. Due to their specific physical properties MMW have low penetration depth (< 1 mm), which means that the main potential target of these frequencies are keratinocyte that are the main constituent of the first layer of skin (Orlacchio et al., 2019; Zhadobov et al., 2011). In MMW's studies several experiments of microarrays were performed to evaluate the effect of these frequencies (30 – 300 GHz) on gene expression (Habauzit et al., 2014a; Le Quément et al., 2012; Millenbaugh et al., 2008;

Soubere Mahamoud et al., 2016). A pioneering study was performed to analyse the heat-associated effect of high power MMW exposure on rat skin and compare to conventional heat stroke. This experiment evidenced that the main effect was the heat effect associated to the MMW (Millenbaugh et al., 2008). Some other experiments have tried to discriminate between thermal and non-thermal effects. First of all, these approaches did not evidenced specific action of these frequencies on cellular stress derivate functions (Le Quément et al., 2014). Microarray analysis of primary cultures of keratinocyte exposed to non-thermal 60 GHz millimeter wave (incident power density (IPD) of 1.8 mW/cm<sup>2</sup>) has enabled to identify very few differentially expressed genes (Le Quément et al., 2012). No effects were observed under non-thermal exposure even with higher IPD (Habauzit et al., 2014b; Soubere Mahamoud et al., 2016). If MMW does not have any direct effect, the question of co-exposure was to be explored. At the former ICNIRP (Ahlbom et al., 1998) IPD limit (20 mW/cm<sup>2</sup>), our group have previously showed that some slight effects could be evidenced in co-exposure scenario (Habauzit et al., 2014a; Le Quément et al., 2012; Soubere Mahamoud et al., 2016). Indeed, when MMW exposure is associated with another stressor agent, such as a heat shock or as a metabolic stress, around 1% of the all genes modified by the stressor agent are targeted by MMW. Nevertheless, when the co-stressor agent is removed, effects were no more observed to the short-term in *in vitro* models (Habauzit et al., 2014a; Soubere Mahamoud et al., 2016), but also to the long-term exposure in animal models (Habauzit et al., 2020). Among the 1% genes modified when MMW are applied with the co-stressor agent heat shock, 3 genes (*ADAMTS6*, *IL7R* and *Nog*) were evidenced as reproducibly differentially expressed in a primary culture of keratinocytes (Habauzit et al., 2014b). It is important to note that these results were not confirmed by using three other different primary cultures and one HaCaT cell line (Martin et al., 2020). Taken together, these results showed that the

transcriptional response to MMW was of very low amplitude and could be variable from one cellular model to another.

In order to get deeper insight into transcriptional changes induced by millimeter-wave and to identify potential biomarkers of exposure, we performed a gene expression analysis of three distinct models of human keratinocytes exposed to 60-GHz millimeter-waves by using Bulk RNA barcoding sequencing (BRB-Seq). These experiments focus on the molecular aspect of the cellular response to MMW, so we have chosen *in vitro* models which are perfectly suit to this purpose and which have the advantage to fit the 3R principle and replace animal model (Russell and Burch, 1959).

## **2. Materials and methods.**

### *2.1. Cell cultures.*

Three cell types were used for this study. Two different primary cultures of human keratinocyte cells which are pools issue from 3 donors and are named HEK (Invitrogen, Saint-Aubin, France) and NHEK (Lonza, Levallois-Perret, France). HEK and NHEK were cultured and exposed as previously described (Habauzit et al., 2014a) and used between passages 4 and 9. In parallel, the keratinocyte cell line HaCaT was used between passages 5 and 10. They were maintained and exposed as previously described (Le Quément et al., 2014). All experiments were replicated independently between 3 to 6 times.

### *2.2. Exposure system and experimental setup.*

The exposure system is described in Zhadobov et al. (Zhadobov et al., 2007). Briefly, the near-field irradiation (Zhadobov et al., 2012) was performed from the bottom of the 6-well plate, placed in MEMMERT UNE400 incubator, in order to ensure the complete and relevant 60.4 GHz exposure to the former ICNIRP guideline limit of exposure (Ahlbom et al., 1998) of 20 mW/cm<sup>2</sup> and to the new ICNIRP's exposure limit in occupational exposure scenario ("Guidelines for Limiting Exposure to Electromagnetic Fields (100 kHz to 300 GHz)," 2020) of 10 mW/cm<sup>2</sup> or 5 mW/cm<sup>2</sup> as previously described (Habauzit et al., 2014a). Before exposure, the medium was renewed with Serum-Free Media (SFM) supplemented with 20 mM of Hepes for primary culture and specific DMEM medium for HaCaT cells (Le Quément et al., 2014; Martin et al., 2020). The exposure was performed during 3 hours or 14 hours (overnight) depending upon the experiment conditions. Three conditions were used: Sham unexposed cells (Sham), MMW exposed Cells (Expo), and heat shock control (HSC) as the IPD is high enough to increase temperature in the medium. All conditions were randomly performed in the same incubator with the generator on or off, and for heat shock control incubator consign was increase to mimic the MMW associated heat effect as previously described (Habauzit et al., 2014a). During each exposure, the medium temperature was monitored with 4-channel Reflex fiber optic thermometer (NEOPTIX, Quebec, Canada) and temperature dynamics were equivalent in HSC and Expo and the steady state mean of temperatures of each condition are reported table 1.

Table 1: Temperature measurement during each experimental conditions and cell used for 3h of exposure.

Conditions	HEK	NHEK	HaCaT
Sham	35.9±0.2 °C	36.2±0.24 °C	35.1±0.39 °C
HSC	40.8±0.38 °C	40.9±0.2 °C	40.82±0.67 °C
Expo	40.5±0.6 °C	40.5±0.15 °C	40.8±0.47 °C

### 2.3. RNA extraction and Bulk RNA barcoding sequencing (BRB).

RNAs were extracted with the Macherey-Nagel NucleoSpin RNA kit (Macherey-Nagel, Hoerdt, France), and then quantified by NanoDrop 8000 spectrophotometer (NanoDrop technology, Cambridge, UK). The library was prepared by using the 3' Bulk RNA Barcoding and Sequencing (BRB-seq) protocol (Alpern et al., 2019). Briefly, 4  $\mu$ L total RNA with a concentration of 2.5 ng/ $\mu$ L was used for initial reverse transcription and template switching. 50 ng of double-strand cDNA tagmentation was performed with Nextera XT kit (Illumina, #FC-131-1024), according to manufacturer instruction. The library was sequenced on Illumina HiSeq 4000 sequencer as Paired-End 100 base reads following Illumina's instructions by the GenomEast Platform. Image analysis and base calling were performed using RTA 2.7.7 and bcl2fastq 2.17.1.14. Adapter dimer reads were removed using DimerRemover [DimerRemover: <https://sourceforge.net/projects/dimerremover/>]. GenomEast was used as previously described for quality control and preprocessing of data (Draskau et al., 2021).

#### *2.4. Preprocessing of BRB-seq raw data*

As previously described (21), a pipeline was used: 1) to verify the correspondence of the 16 bases of the first reads, and all must have a quality score >10, with a specific barcode. The unique sample-specific barcode corresponds to the first 6 bp, and the unique molecular identifier (UMI) corresponds to the following 10 bp; 2) to align second reads to the human reference transcriptome (UCSC website, hg38, 2019/12) (22). Following quality controls and data preprocessing, the resulting count matrix was normalized by using regularized the rlog method implemented into the DESeq2 package (23). Sequencing data and processed data were deposited at the GEO repository under the accession number GSE234710.



## 2.5. Differential gene expression analysis

The following statistical comparisons were made in the AMEN suite of tools (Chalmel and Primig, 2008) : 1) Sham conditions of all keratinocyte models were compared; 2) Sham vs Expo 20 mW/cm<sup>2</sup> vs HSC equivalent to 20 mw/cm<sup>2</sup> treated during 3 hours. Comparison were performed for NHEK, HEK and HaCaT cell models; 3) Sham vs Expo (5, 10 and 20 mW/cm<sup>2</sup>) vs HSC (temperature equivalent to 5, 10, and 20 mW/cm<sup>2</sup>) treated during 3 hours, in HaCaT cell lines; 4) Sham vs Expo 10 mW/cm<sup>2</sup> and HSC equivalent to 10 mW/cm<sup>2</sup> treated overnight in HaCaT. Briefly, for each comparison, genes showing an expression signal higher than a given background cutoff (corresponding to the overall median of the rlog-transformed UMI dataset, =1.29) and at least a 1.5-fold change were selected. The linear models for microarray data (LIMMA) package was used (F-value adjusted with the false discovery rate method,  $p \leq 0.05$ ) to select genes showing a significant differential expression (Smyth, 2004).

## 2.6. RNA extraction and RT-QPCR analysis validation.

RNA was extracted with a NucleoSpin RNA kit (Macherey-Nagel, Hoerd, France) and then quantified by a NanoDrop 8000 spectrophotometer (NanoDrop Technology, Cambridge, UK). Five hundred nanograms of RNA was reverse-transcribed with an iScript kit (Bio-Rad, Hercules, CA) according to the supplier's instructions. All primers used are presented in Table 2. Two housekeeping genes (*TBP* and *GAPDH*) were used for normalization. QPCR was performed on a Bio-Rad CFX 384-well apparatus with SYBR Green Supermix (Bio-Rad, Hercules, CA).

Table 2: PCR primer used for the RT-PCR validation of the heat shock protein identified in the BRB-sequencing experiments

Gene name and symbol	Forward primer	Reverse primer
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<i>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</i>	<i>TGCACCACCAACTGCTTAGC</i>	<i>GGCATGGACTGTGGTCATGAG</i>
<i>TATA-box binding protein (TBP)</i>	<i>TGCACAGGAGCCAAGAGTGAA</i>	<i>CACATCACAGCTCCCCACCA</i>
<i>Heat shock protein 70 kDa (HSP70A1A)</i>	<i>AGGCCGACAAGAAGAAGGTGCT</i>	<i>TGGTACAGTCCGCTGATGATGG</i>
<i>Heat shock protein 27 kDa (HSPB1)</i>	<i>CAGGACGAGCATGGCTACATCT</i>	<i>GGGATGGTGATCTCGTTGGAC</i>

### 2.7. Statistical analysis.

Statistical analyses for the comparison of conditions were performed using a one-ANOVA test with Tukey's multiple comparisons test on GraphPad Prism software. A difference at  $p$ -value  $\leq 0.05$  was considered statistically significant.

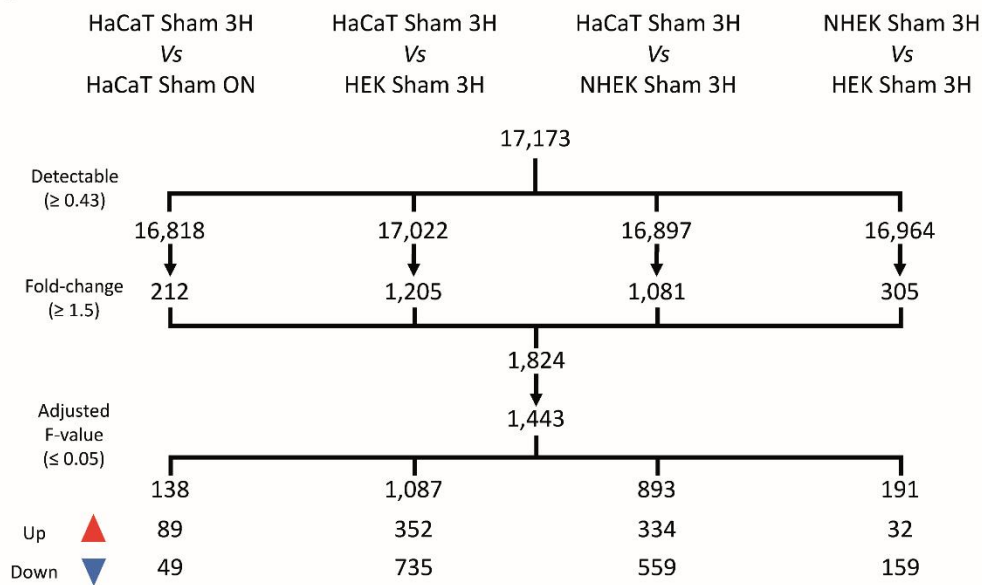
## 3. Results

### 3.1. Gene expression differences between cell models and time courses

In order to validate the methodology used, we compared the basal gene expression between keratinocyte derived cells. Using the BRB-seq data obtained with the sham samples, we were able to observe a clear dichotomy on the hierarchical clustering of the 1,443 genes differentially expressed between each cell model (Table S1). Indeed, we observed gene expression changes between the HaCaT cell line (sham exposed during 3 hours or Overnight (ON, 14 hours treatment) and the primary culture of keratinocytes (Fig. 1, panel A). The 1,443 differentially expressed genes (DEGs) were subsequently partitioned into six clusters (named C1 to C6; Fig. 1, panel B). Cluster revealed that C1 and C6 illustrate transcriptional differences between primary cultures (HEK and NEHK) and cell lines (HaCaT). Genes up-regulated in the primary cultures (C1) are significantly associated with epidermis development (58 genes,  $p$ -value =  $4.4 \times 10^{-23}$ ), keratinocyte differentiation (46 genes,  $p$ -value

=  $2.1 \times 10^{-21}$ ), epidermal cell differentiation (49 genes, p-value =  $6.6 \times 10^{-21}$ ) (Fig. 1; panel B; Table S2), as confirmed by KEGG analysis. It is important to note that the HaCaT also express these specific genes which confirms the classification of this cell line as keratinocyte. We can remember that HaCaT has been described as immortalized cells able to proliferate, but with all differentiated properties of natural keratinocytes (Boukamp et al., 1988). On the other hand, genes down-regulated in primary cultures (C6) are associated with GO terms such as cell cycle (123 genes, p-value =  $1.0 \times 10^{-29}$ ), DNA replication (38 genes, p-value =  $4.5 \times 10^{-17}$ ), and KEGG terms such as DNA replication (16 genes, p-value =  $1.3 \times 10^{-115}$ ) mismatch repair (8 genes, p-value =  $1.4 \times 10^{-8}$ ), and nucleotide excision repair (7 genes, p-value = 0.0043). These enrichments are consistent with the higher proliferative status of the HaCaT cell line compare to the primary keratinocyte cultures (Fig. 1, panel B). Interestingly C5 is associated with the infection and cell reaction toward virus infection (Fig. 1, panel B) – confirming that it is safer to handle established cell lines than primary cultures from donors. Genes belonging to C3 and C4 display include genes showing slight gene expression changes over culture duration (3h and ON) in the primary culture (HaCaT) - probably related to cell confluence. No terms were found to be enriched in C3 and C4.

A



B

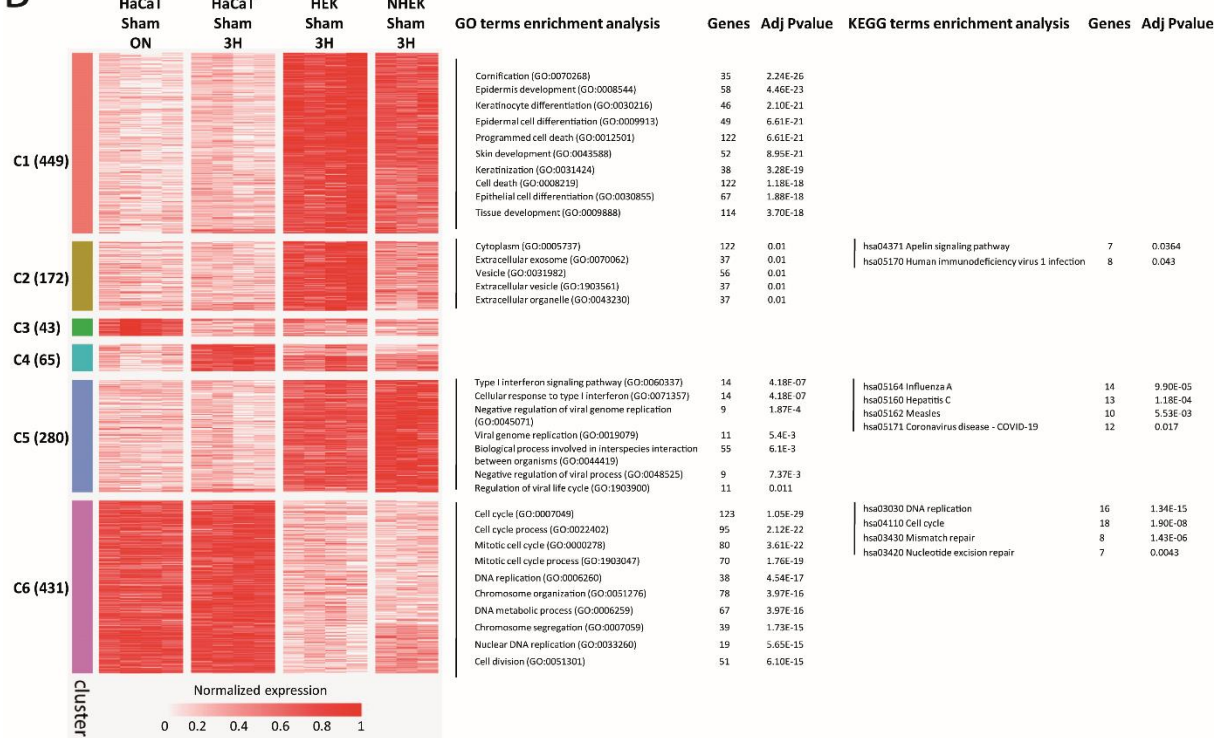


Fig. 1: Transcriptional landscape of sham condition of the different human keratinocyte models. A) Differentially expressed genes (DEGs) between each cell types used, analyzed by BRB-seq by comparing HaCaT Sham control 3h vs HaCaT sham control at 14h, HEK sham control at 3h, and NHEK Sham control at 3h. A total of 1,443 DEGs were detected by applying 3 filtration steps for each comparison: (i) the detectable cutoff (0.43); (ii) the fold-change cutoff (1.5) and (iii) the adjusted F-value (0.05). B) Heatmap representation of DEGs during human keratinocyte cell models revealed by BRB-seq through comparisons of control conditions. In total, 1,443 DEGs were detected and grouped into 6 expression patterns (C1–C6). Each row represents a gene and each column a time point. The color code indicates scaled gene expression values between 0 and 1. GO terms and KEGG enrichment analysis revealed biological processes terms significantly associated with each expression pattern, which are indicated on the right with associated number of genes and p value. Besides, enriched cell types based on corresponding markers are provided for each pattern.

### *3.2. Delineation of the thermal from the electromagnetic effect on the three cell populations after 3h of treatment at an Average IPD of 20 mW/cm<sup>2</sup>, or an equivalent heat shock*

The same exposure condition (60.4 GHz at 20 mW/cm<sup>2</sup> during 3h) was independently applied to the three different cells (Fig. 2A). The MMW exposure induced an increase of the medium temperature in the three experimental designs (Table 1), so heat shock controls were performed. As previously shown in table 1, the final temperature was around the same in Expo and HSC conditions. The different experimental conditions have no effect on the cell viability and so no variation in mRNA quantity was observed between conditions and cell types.

Even if there are great differences in the gene expression of the keratinocyte models, the effect of MMW is quite similar and very modest. While considering the comparison Sham-HSC and Sham-Expo, the gene expression varies marginally depending on the cell model, with respectively 0, 4, and 14 modified genes for NHEK, HEK, HaCaT cells (Fig. 2 B-D). All modified genes are linked to the heat effect induced by the MMW and to the heat associated to HSC exposure. DEGs were identified only in HEK and HaCaT cell cultures. With the exception of DDX5 which is down-regulated in HaCaT, all the other DEGs were found to be up-regulated (Fig. 2, panels E, F). It is noteworthy that only DNAJB1 and HSPA1B, two heat shock sensitive genes, were common between these two lists. The KEGG pathway illustrated that these two gene lists are significantly associated with pathway "Protein processing in endoplasmic reticulum" (HSA04141) (4 genes, p-value =  $6.6 \times 10^{-5}$  for HEK and 7 genes, p-value =  $6.7 \times 10^{-8}$  for HaCaT). While comparing just the electromagnetic effect independent from the heat effect (Expo vs HSC), we found that only HSP1AB in HEK was

significantly differentially expressed between HSC and Expo (Fig. 2, panel C). No DEGs were detected in the NHEK cell culture.

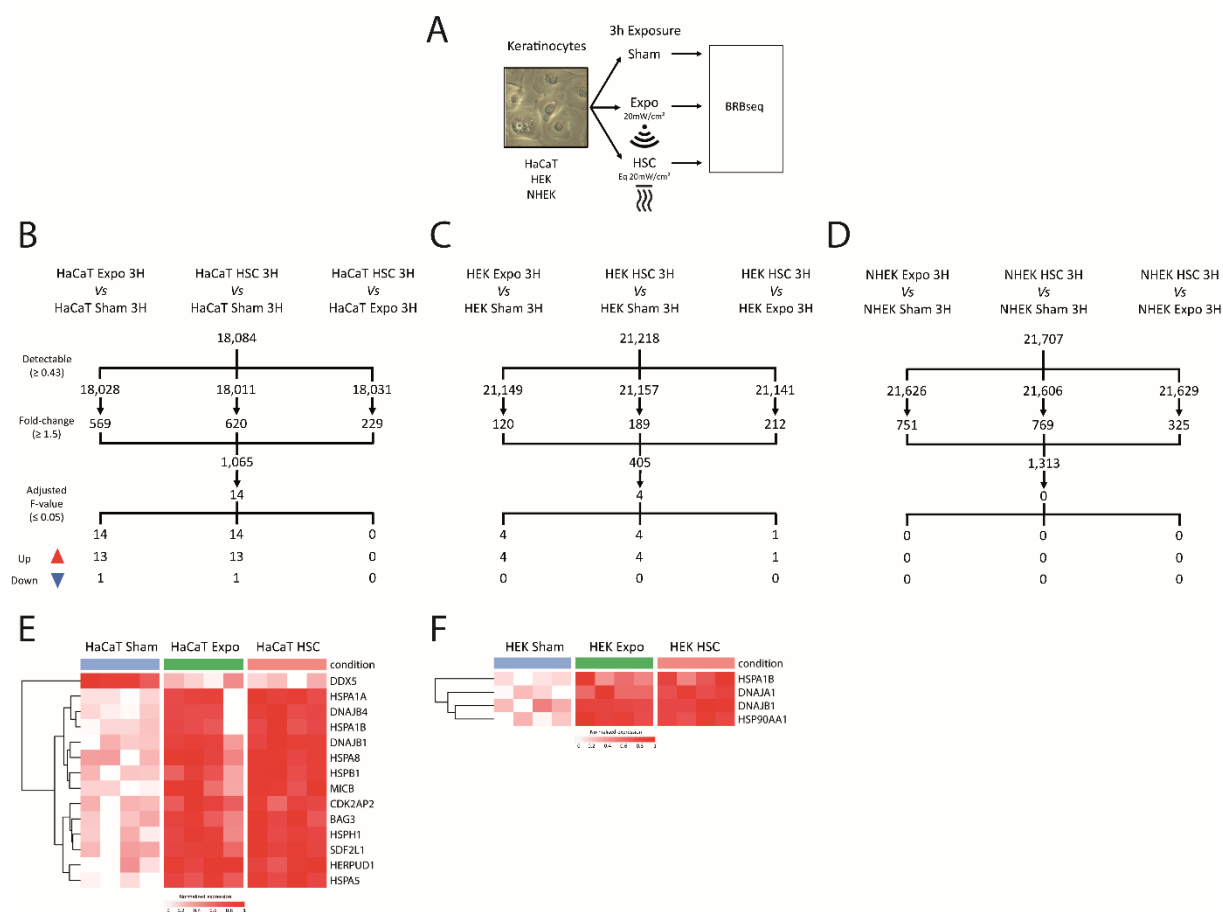


Fig. 2: Experimental protocol and differentially expressed genes. A) Two primary culture of keratinocytes and one keratinocyte cell line were Sham, or millimeter wave (20 mW/cm<sup>2</sup>- Expo), or Heat (with temperature equivalent to 20 mW/cm<sup>2</sup>- HSC) exposed for 3 hours and then analyzed by BRB-seq. Differentially expressed genes (DEGs) between each cell types used, analyzed by BRB-seq by comparing all condition in respectively HaCaT (B), HEK (C) and NHEK (D). Heatmap representation of DEGs during exposition revealed by BRB-seq through comparisons sham, Expo and HSC for the 14 DEG in HaCaT (E), and 4 DEG in HEK (F).

In order to get deeper insight in the effect of MMW on the gene expression we decided to select two genes impacted by the treatment, HSPA1A/B (also known as HSP70) and HSPB1 (HSP27). The PCR validation showed that HSPA1A/B were amplified and overexpressed by exposure in the two primary cultures, and in the HaCaT cells (Fig. 3, panels A, B and C). Moreover, the absence of significant difference between HSC and Expo was confirmed in any

of the three cell models. The HSPB1 gene was found to be differentially expressed only in HaCaT cells, with a significant effect on HSC condition and a trend for the Expo (Fig. 3, panel F). No significant expression changes were observed in the two primary cultures as shown by the BRB-seq approach (Fig. 3, panels D and E).

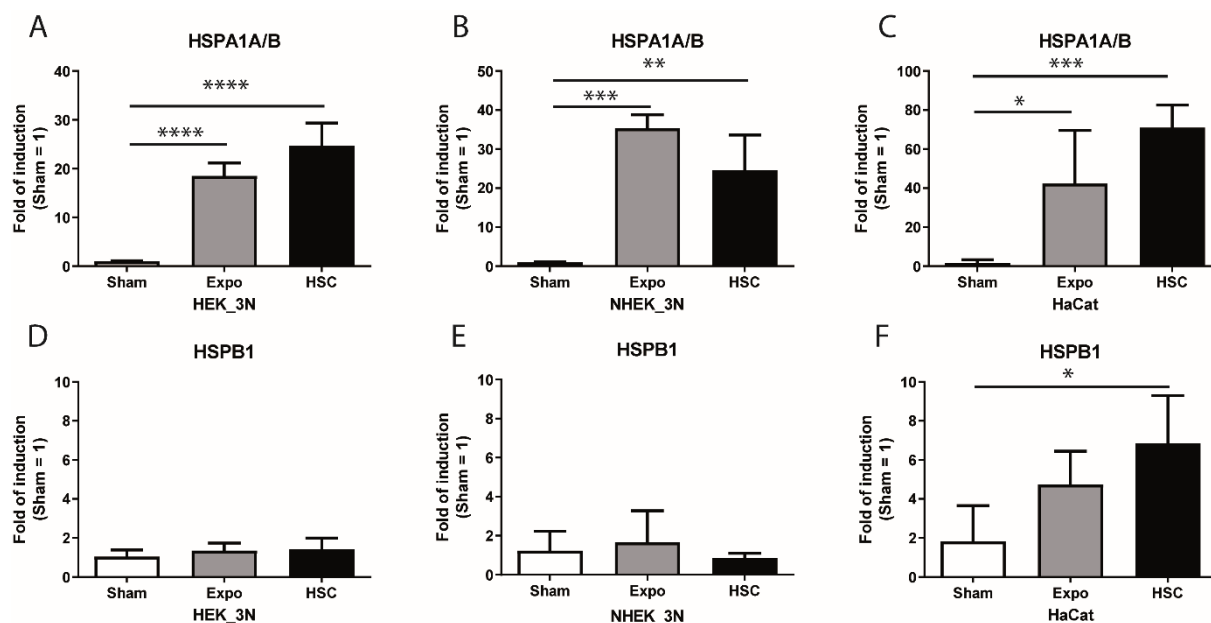


Fig. 3 : Evaluation of the treatment (MMW or Heat) on the expression of the HSPA1A/B and the HSPB1 in the two primary cultures and the cell line.

### 3.3. Millimeter wave exposure effect on HaCaT cell line in IPD gradient and medium long-term treatment

A dose effect was achieved by increasing the incident power density (IPD) from 5 mW/cm<sup>2</sup> to 20 mW/cm<sup>2</sup> in our exposure condition. This last IPD correspond to the former maximum allowed IPD for general public in the limit of 1 cm<sup>2</sup> of skin exposure (Ahlbom et al., 1998). However the new ICNIRP limit (10 mW/cm<sup>2</sup>) was also tested (“Guidelines for Limiting Exposure to Electromagnetic Fields (100 kHz to 300 GHz),” 2020). Again, in order to compare

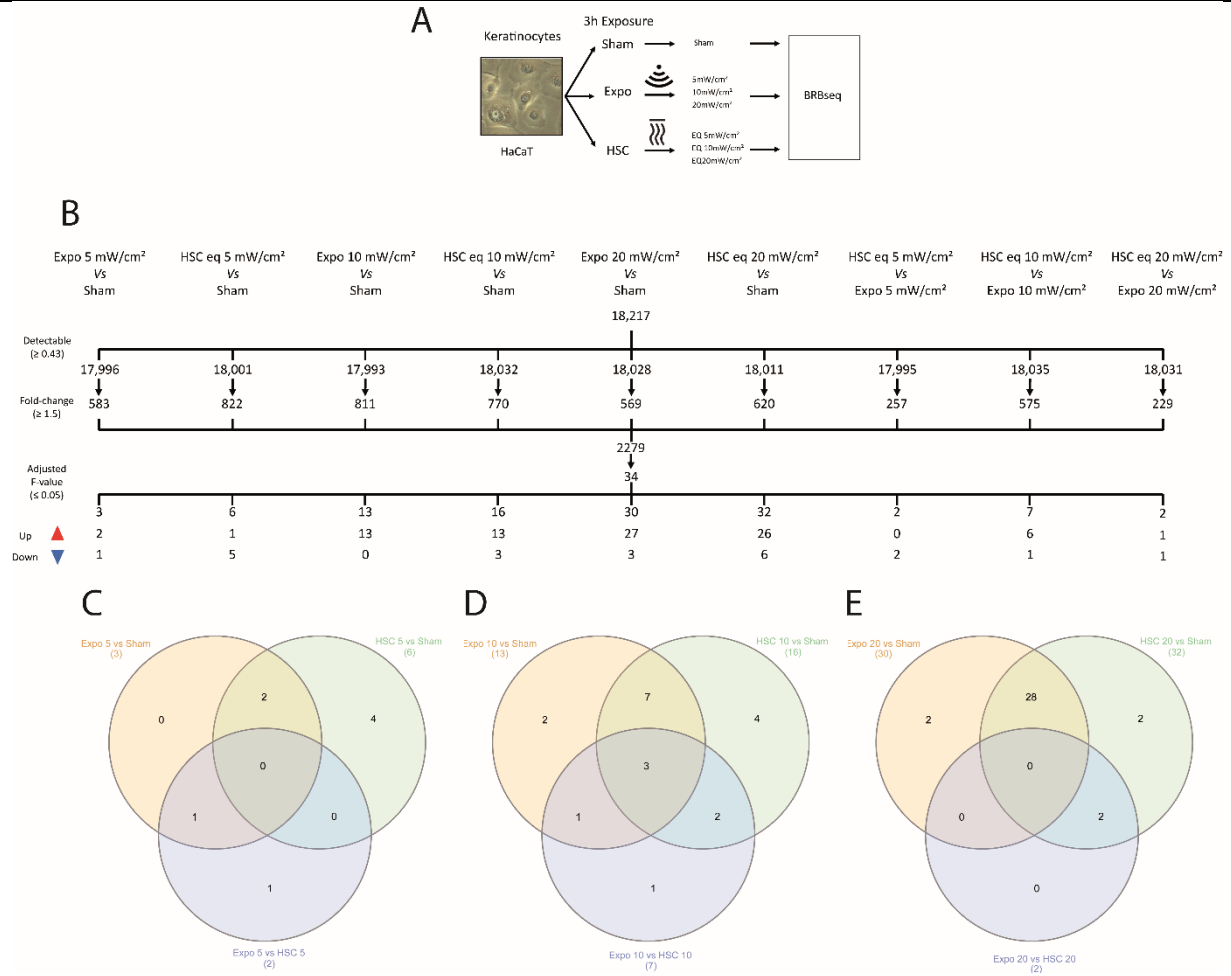
the electromagnetic effect independently from the heat effect, we included dedicated HSC samples which have the same temperature as the exposure at each IPD (Table 3). The evaluation was performed on the HaCaT cells, and we first focused on a 3h exposure (Fig. 4, panel A). In total, 34 genes were found to be significantly differentially expressed and a functional analysis revealed a significant association with chaperone-mediated protein folding (10 genes,  $p\text{-value} = 4.2 \times 10^{-14}$ ), and response to heat (9 genes,  $p\text{-value} = 5.9 \times 10^{-8}$ ). These results proved that the main observed effect is the heat effect in the both condition Expo and HSC (Fig. 4, panel B; Fig. 5). While most of these DEGs shared a common differential expression between the Sham condition and both Expo and HSC conditions, few of them are differently expressed between the Expo and HSC at 5 mW/cm<sup>2</sup> (2 genes: *CGAS*, *SDF2L1*), at 10 mW/cm<sup>2</sup> (7 genes: *CCDC117*, *DNAJB1*, *HSPA1B*, *KRT17*, *MKNK2*, *SEL1L*, *TUBB3*) and, at 20 mW/cm<sup>2</sup> (2 genes: *CLU*, *PLK2*) (Fig. 4, panel C-E). Again, most of these genes are associated with the heat effect and could illustrate the small difference in the way cells are warmed (electromagnetic fields vs convection). Apart from the cell-cycle *PLK2* gene for which the expression is maintained during an exposure at 20 mW/cm<sup>2</sup>, most of the other DEGs display similar expression patterns between Expo and HSC conditions as shown in Fig. 5. Altogether, these results show a lack of athermic electromagnetic effect on HaCaT cell line gene expression while they are exposed up to 20 mW/cm<sup>2</sup>.

Table 3: Temperature measurement during each exposure in HaCaT doses exposure.

Exposure duration	Sham	Expo 5 mW/cm <sup>2</sup>	HSC Equivalent 5mW/cm <sup>2</sup>	Expo 10 mW/cm <sup>2</sup>	HSC Equivalent 10 mW/cm <sup>2</sup>	Expo 20 mW/cm <sup>2</sup>	HSC Equivalent 20 mW/cm <sup>2</sup>
3 Hours	35.1±0.39 °C	36.3±0.5 °C	36.55±0.53 °C	38.3±0.41 °C	38.78±0.95 °C	40.8±0.47° C	40.82±0.67 °C
14 Hours	35.77±0.23° C			38.7±0.69 °C	38.87±0.7 °C		



ON



**Fig. 4: Experimental protocol and differentially expressed genes.** A) HaCaT keratinocyte cell line was Sham, or millimeter wave- (At an IPD of 5, 10, and 20 mW/cm<sup>2</sup>- Expo 5-20 mW/cm<sup>2</sup>), or Heat- (with temperature equivalent to 5, 10, and 20 mW/cm<sup>2</sup>- HSC eq 5-20 mW/cm<sup>2</sup>) exposed for 3 hours and then analyzed by BRB-seq. Differentially expressed genes (DEGs) between each conditions used, analyzed by BRB-seq by comparing all condition in respectively against Sham control and then each Expo vs HSC conditions were compared. Venn diagrams represent the gene repartition between Expo and HSC vs Sham and Expo vs HSC conditions respectively at 5 (C), 10 (D), and 20 (E), mW/cm<sup>2</sup> or equivalent temperature.

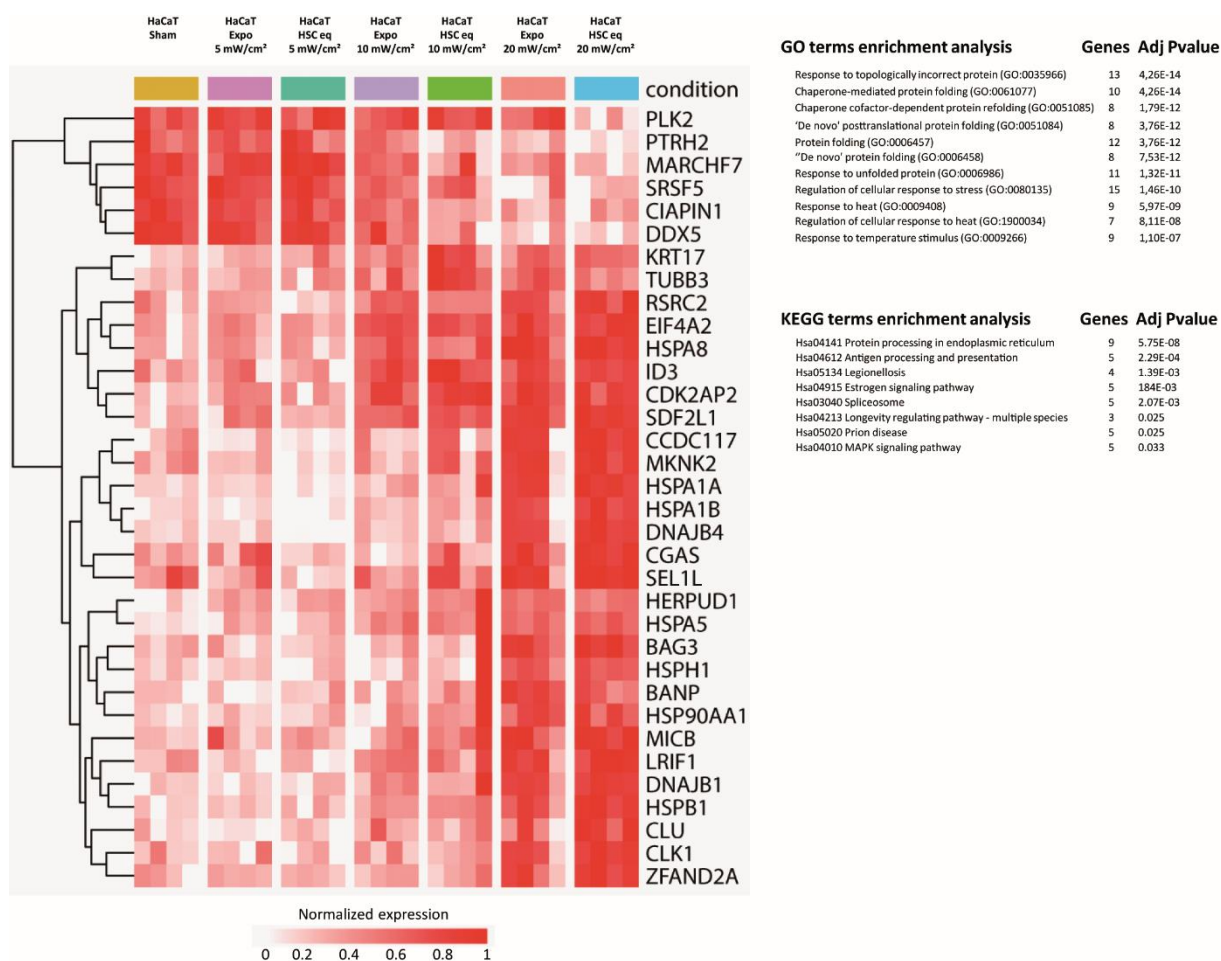
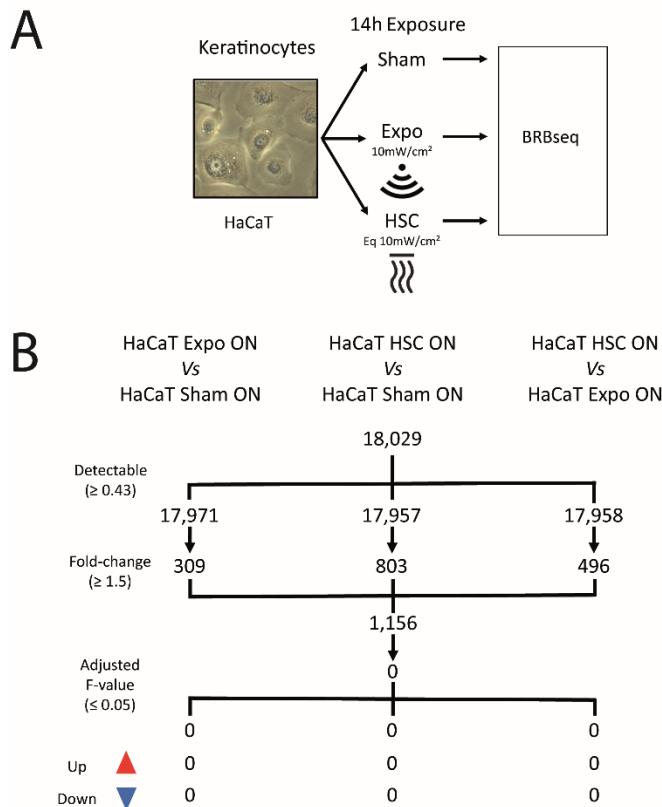


Fig. 5: hierarchical clustering representation of DEGs during HaCaT cell lines sham, or millimeter wave- (At an IPD of 5, 10, and 20 mW/cm<sup>2</sup>- Expo 5-20 mW/cm<sup>2</sup>), or Heat- (with temperature equivalent to 5, 10, and 20 mW/cm<sup>2</sup>- HSC eq 5-20 mW/cm<sup>2</sup>) exposed. In total, 34 DEGs were detected. Each row represents a gene and each column a time point. The color code indicates scaled gene expression values between 0 and 1. GO terms and KEGG enrichment analysis revealed biological processes terms significantly associated with each expression pattern, which are indicated on the right with associated number of genes and p value.



*Fig. 6: Experimental protocol and differentially expressed genes. A) HaCaT keratinocyte cell line was Sham, or millimeter wave at 10 mW/cm<sup>2</sup> (Expo 10 mW/cm<sup>2</sup>), or Heat- (with temperature equivalent to 10 mW/cm<sup>2</sup>- HSC eq 10 mW/cm<sup>2</sup>) exposed for 14 hours (overnight\_ON) and then analyzed by BRB-seq. Differentially expressed genes (DEGs) between each conditions used, analyzed by BRB-seq by comparing all condition in respectively against Sham control and then each Expo vs HSC conditions were compared.*

The second HaCaT exposure focused on an extend exposure duration (14h, overnight) to a 10 mW/cm<sup>2</sup> IPD (Fig. 6 pattern A and B). The overall temperature differences between the exposure and the HSC equivalent were close enough to delineate the only effect of the electromagnetic field independent from the MMW associated heat effect. The statistical analysis was not enabled to identify DEGs when considering at medium-long term exposure to MMW (14 hours).

#### 4. Discussion

Millimeter waves are currently becoming an area of interest due to their future use in several applications. These frequencies have very low penetration depth, making the skin

and retina, the main biological targets. As the electromagnetic energy is absorbed by the first cells layers, the local SAR can be high, even for relatively low incident power density. For MMW, the only proven effect is the increase of sample's temperature, which appears for IPD higher 5 mW/cm. Few transcriptomic studies have been dedicated to MMW. Most of them reported that short-term acute exposure had no impact on gene expression, at least as long as the conditions are non-thermal (Habauzit et al., 2014a; Le Quément et al., 2012; Soubere Mahamoud et al., 2016). However, some studies have shown that a few rare genes may be sensitive to MMW exposure, but the reproducibility of this observation could not be fully verified (Habauzit et al., 2014a; Lawler et al., 2022; Le Quément et al., 2012; Millenbaugh et al., 2008; Soubere Mahamoud et al., 2016). This problem is probably related to the exposure conditions and the keratinocyte cell models used (Habauzit et al., 2014a; Martin et al., 2020). Therefore, our idea was to evaluate the gene expression of different skin derivate models in several MMW-exposure scenarios. For that purpose, the BRB-seq technology was used to compare first the transcriptional landscape of different keratinocytes models. On the thousands of genes showing gene expression changes, some of them were found to be significantly differentially expressed (1443 genes). This result has validated differences in our keratinocyte cell models and have allowed us to verify that the genes differentially expressed between skin models were associated with pathways involved in cell differentiation and cell proliferation. The comparison of expression profiles between several keratinocyte cell models allowed us to technically validate our approach.

Then considering the impact of MMW exposure on gene expression, a very low number of gene was finally observed in our keratinocyte models. Primary cultures of keratinocytes

were only slightly sensitive to MMW and HSC treatment with 0 and 4 DEG for exposure at the ICNIRP former limit. The HaCaT cell line is barely more sensitive to the same treatment, with 14 DEG. In HaCaT cell line, a gradient of MMW exposure induces modification of only 34 genes, with majority of them related to heat shock response or to protein denaturation. Interestingly, the higher the IPD, the higher the number of genes found, in relation to the temperature rise. Another important point is that DEGs in the Expo condition are generally also found in the heat shock control indicating that the cell reaction is mainly due to the temperature increase induced by the high IPD used (corresponding to the maximum ICNIRP limit). Even if a lot of genes shows a fold change upper than 1.5, very few evidence a significant difference in their expression. Then considering the differentially expressed gene, the quantitative variation is always against the Sham control. Finally, no quantitative difference is observed between Expo and HSC as shown in all tables. While some genes have been found to be differentially expressed between Expo and HSC conditions, they are all known to be related to response to heat. These gene expression changes might thus be explained by differences between conventional heating and heating caused by the rotation of free water molecules (MMW). Thus, the current study did not evidence any specific non-thermal effects of the electromagnetic field, regardless of the IPDs or of the exposure.

In parallel, no effects were identified when considering medium-long term exposure (14h) to MMW at 10 mW/cm<sup>2</sup>, suggesting that the cells had been able to adapt and so had managed to return to an equilibrium identical to the control situation. The same type of observation was described by Le Quement and collaborators (Le Quément et al., 2012), where the differential in expression was much smaller after 24 hours of exposure, compared with 6 hours. It is possible that the duration and/or the mode of exposure is an important parameter of variation. A recent publication with prolonged repeated exposures (5h per day

for 2 and 4 days) on human fibroblasts showed an effect of a 60 GHz exposure on gene expression (Lawler et al., 2022). This study used an IPD of 2.6 mW/cm<sup>2</sup>, which in their culture system generated a temperature increase of 1.2°C that is close to the one we obtained in the current study. It is possible that intermittent exposure over several days has a more pronounced effect than continuous exposure. It will be interesting in the future to systematically compare these different modes of exposure.

It can be noted that the genes highlighted in our previous studies (Habauzit et al., 2014b; Le Quément et al., 2012; Soubere Mahamoud et al., 2016) were not identified in the current study. As this previous work was done on primary cultures from other donors, it is reasonable to assume that it may reflect the heterogeneity of the response from one cell model to another. The other important information to be drawn from this study is that the effect of exposure on gene expression is strongly correlated with thermal effects. At least, in the context of *in vitro* studies, with short exposures. No more effects were observed in neuron like cells PC12 (Haas et al., 2017, 2016a, 2016b). Long term *in vivo* exposure of rat skin to an incident power density of 10 mW/cm<sup>2</sup>, 3 h per day and 3 days per week for 5 months to a frequency of 95 GHz in duplicate have not allow to show any difference in gene expression in athermic condition (Habauzit et al., 2020). These results support previous findings and show that acute exposure of short duration does not induce intense cellular stress that requires an adaptive transcriptional response from the cells. However, the question of the impact of chronic exposure on whole-organism remains open for millimeter waves especially to a frequency of 60 GHz. It's important to note that our experimental conditions differ from those of the whole organism. Firstly, it is quite possible that thermal effects are overestimated in an *in vitro* system, as our cellular model lacks the thermolysis-regulating system provided by superficial blood flow (Alekseev et al., 2005). Secondly, the

hypothesis of an indirect physiological effect due to superficial heating of the skin cannot be ruled out. For example, recent findings indicate that radiofrequency exposure can trigger some physiological response associated with thermoregulation and cold-perception in rats (Mai et al., 2021, 2020).

However, the search for physiological indirect effects is not the aim of this study, and this point will require further specific studies. Through our BRB-seq approach, we sought for direct effects, hoping that exposure would induce transcriptional changes that could point us in the direction of adaptive mechanisms and associated signaling pathways.

## **5. Conclusion**

In this study, several exposure scenarios were explored using an innovative barcode sequencing method. Although some genes were identified during these evaluations, they were mainly due to small differences in the heating method, convection versus radiation. They highlight that, apart from a heat effect associated with these waves, there does not appear to be any electromagnetic effect alone during acute exposure. Lot of information are still missing in the evaluation of the MMW effect on living, several other cell types should be evaluated such as Melanocytes, Langerhans, and Merkel cell but also some neuron like cells that are connected to skin. Ex vivo skin model should be therefore used for a better understanding of the MMW effect on living and these kinds of model are more integrated model. Finally, in order to explain certain different effects depending on the individual, studies on potential chronic effects but also epigenetic effects should be explored.

## Author contributions

Conceptualization, Data curation, Formal analysis, Resources Roles/Writing - original draft: DH, YLD, FC; Funding acquisition, Supervision, Project administration: DH; Investigation, Methodology: CM, BE, FP, KR, TD, AL, DH; Software: FC, TD, AL; Exposure System: MZ, RS, DH; Validation: CM, DH, Writing - review & editing: YLD, MZ, AL, FC, DH

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors gave their consent for publication.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data are available in GEO repository under the accession number GSE234710

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### **Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



## Highlights

Millimeter waves have no effect on Keratinocyte models transcription

The only recognized effect is the heat effect associated to MMW

The only effects are due to the heat generation process